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Chicken interleukin-21 is costimulatory for T cells and blocks maturation of dendritic cells

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ABSTRACT

In mammals, interleukin-21 (IL-21) is an immunomodulatory cytokine with pleiotropic effects on the proliferation, differentiation and effector functions of T, B, NK and dendritic cells. A cDNA encoding the chicken orthologue of IL-21 (chIL-21) was cloned by RT-PCR from RNA isolated from activated chicken splenocytes and consists of 438 nucleotides, encoding an open reading frame of 145 amino acids (aa). Chicken IL-21 has 20–30% aa identity to its orthologues in mammals, *Xenopus* and fish, but is more highly conserved within Aves (50–80%). The four alpha-helical bundle structure of mammalian IL-21 appears to be conserved in the predicted chicken protein, as are the four cysteine residues required for the formation of two disulphide bridges. A glutamine residue in aa position 129, which has been implicated in the binding of IL-21 to the IL-2 receptor γ -chain in mammals, is also conserved. ChIL-21 is expressed in most lymphoid tissues, predominantly by CD4⁺ TCR $\alpha\beta$ ⁺ T cells. As in mammals, chIL-21 synergistically enhances T-cell proliferation and inhibits maturation of dendritic cells.

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1. Introduction

Interleukin-21 is a type I cytokine, belonging to the four α -helical bundle family of cytokines, with structural and functional homology to IL-15, IL-2 and IL-4, and was first described by Parrish-Novak et al. (2000). In mammals it is an immunomodulatory cytokine produced by CD4⁺ T and natural killer T (NKT) cells, and has pleiotropic effects on the proliferation, differentiation and effector functions of T, B, natural killer (NK) and dendritic cells (DCs) (reviewed by Leonard et al., 2008; Mehta et al., 2004 and Spolski and Leonard, 2008). These effects are exerted following ligation of the ligand with its receptor, composed of a specific receptor (IL-21R) (Ozaki et al., 2000) and the common cytokine receptor γ chain (γ_c), resulting in activation of the Jak-STAT signal transduction pathway (Asao et al., 2001; Habib et al., 2002). Depending on the cell context and cytokine environment, the actions of IL-21 can be immunostimulatory (Parrish-Novak et al., 2000; Zeng et al., 2005) or immunosuppressive (Brandt et al., 2003; Spolski et al., 2009). IL-21 also plays a crucial role in B cell function, regulating Ig production (Ozaki et al., 2002), driving differentiation to plasma cells (Ettinger et al., 2005; Ozaki et al., 2004), or inducing

B cell apoptosis (Jin et al., 2004; Mehta et al., 2003). In conjunction with this, IL-21 plays an important role in the generation of T follicular helper cells (Tfh) and germinal centre development (reviewed by Spolski and Leonard, 2008, 2010). IL-21 is also a critical regulator of Th17 development (Monteleone et al., 2008).

Until recently, our understanding of the repertoire of immune mediators, including cytokines, chemokines, toll-like receptors, etc. in the chicken, when compared to mammals, was limited. However, the release of the chicken genome sequence (International Chicken Genome Sequencing Consortium, 2004) has revolutionised our ability to understand both the repertoire and the biology of these and other immune-related molecules (Kaiser et al., 2005; Kaiser, 2007, 2010). Although many apparent homologues of mammalian immune genes have been identified in the chicken genome, many others appear to be absent (Kaiser, 2007, 2010; Kaiser and Stäheli, 2008), and these differences may explain some of the fundamental differences seen between chickens and mammals in their respective immune systems.

We have previously shown that the Th1–Th2 paradigm extends beyond mammalian species, to the chicken (Degen et al., 2005), suggesting evolutionary conservation of at least these two lineages of effector CD4⁺ T cells. In the last two decades the field of T cell biology has rapidly evolved, and many new effector and regulatory T cell subsets have been described in mammals. Whether equivalent and orthologous populations of cells exist in the chicken remains to be seen, but it is now possible to readily identify, and generate avian-specific reagents to, many of the immune-related molecules necessary to further dissect the immune response in

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the chicken. In this study we describe for the first time the cloning of chicken IL-21 (chIL-21) cDNA, and analyse its expression and bioactivity *in vitro*. This work provides the basis for further studies to investigate if Tfh and Th17 T cell subsets are also present in the chicken.

2. Materials and methods

2.1. Cloning of chIL-21 cDNA

To amplify the full length chIL-21 cDNA, nested primers were designed according to the predicted sequence found in Ensembl (http://www.ensembl.org/Gallus_gallus). The first primer pair (chIL-21_F1: 5'-ACTCATCAGTATGGAGAGGAT-3' and chIL-21_R1: 5'-TCACATTGTCTTAAATAGCATA-3') flanked the predicted cDNA sequence and were used in the first round of PCR. To increase specificity of the PCR, a nested set of primers were designed to the predicted full length cDNA sequence (chIL-21_F2: 5'-ATGGAGAGGATGATTATTTTC-3' and chIL-21_R2: 5'-TCAAATAGTAGATTTTCCGTA-3') for a second round of PCR. Total RNA was extracted from chicken splenocytes stimulated with 1 µg/ml Concanavalin A (ConA) (Sigma, Poole, UK) for 24 h using an RNeasy mini kit (Qiagen, Crawley, UK) following the manufacturer's instructions. First strand synthesis was for 50 min at 42 °C in a 20 µl volume containing 4 pmol of oligo dT, 200 U Superscript II (Invitrogen, Paisley, UK) and 500 ng RNA. After denaturation of the reverse transcriptase at 94 °C for 3 min, 5 µl of this reaction mix were added as template to a 20 µl PCR reaction, containing 100 pmol each of primers chIL-21_F1 and chIL-21_R1, 0.4 mM dNTPs and 1 µl *Taq* polymerase (Invitrogen). Cycling conditions were 1 cycle of 94 °C for 3 min, 3 cycles of 94 °C for 60 s, 60 °C for 30 s and 72 °C for 90 s, 3 cycles of 94 °C for 60 s, 58 °C for 30 s and 72 °C for 90 s, and then 30 cycles of 94 °C for 60 s, 57 °C for 30 s and 72 °C for 90 s. Five µl of the first PCR product were used in a nested PCR, containing 10 pmol each of primers chIL-21_F2 and chIL-21_R2, 0.4 mM dNTPs and 1 µl *Taq* polymerase (Invitrogen) in a 20 µl reaction volume. Cycling conditions were 1 cycle of 94 °C for 3 min and 30 cycles of 94 °C for 60 s, 57 °C for 30 s and 72 °C for 90 s. The resulting PCR product was ligated into pGEM-T Easy vector (Promega, Southampton, UK), and the complete sequence of three clones determined on each strand. An *EcoRI*-fragment containing chIL-21 was sub-cloned into the eukaryotic expression vector pCI-neo (Promega), giving rise to pCI-neo-chIL-21. The cDNA sequence has been submitted to Ensembl with the accession number AM773757.

2.2. Tissues and cells

Tissues and splenocyte subsets were obtained from three specified-pathogen-free (SPF) 6-week old outbred Rhode Island Red (RIR) chickens. Lymphoid tissues taken were the spleen, bursa, thymus, bone marrow, duodenum, jejunum, ileum, caecal tonsil, Meckel's diverticulum and Harderian gland. Non-lymphoid tissues taken were the kidney, lung, heart, muscle, liver, brain and skin. Splenocyte subsets were isolated as previously described (Wu et al., 2009a). Briefly, chicken spleens were digested with 556 µg/ml DNase I (Roche Diagnostics Ltd., Burgess Hill, UK) and 2.2 mg/ml collagenase D (Roche Diagnostics Ltd.) in Hank's Buffered Salt Solution for 1 h. Single cell suspensions were extracted using Histopaque 1077 (Sigma) followed by three washes in PBS. 10⁸ cells were labelled with mouse anti-chicken CD4, CD8α, CD8β, TCR1 (TCRγδ), TCR2 (TCRαβ1), TCR3 (TCRαβ2), Bu-1, or KUL01 monoclonal antibodies (mAbs) (Southern Biotech, Birmingham, AL, USA). Polyclonal goat anti-mouse IgG-coated microbeads (Miltenyi Biotec, Bisley, UK) were used to isolate labelled cells by standard positive

selection (POSSEL) using an autoMACS™ Pro separator (Miltenyi Biotec).

Splenic T-cell subsets were cultured with or without 1 µg/ml ConA (Sigma), Bu-1+ cells with or without 500 ng/ml PMA (Sigma), and KUL01+ cells with or without 400 ng/ml LPS (*Escherichia coli* serotype 055:B5) (Sigma). Splenic TCRαβ+ cells were also isolated from four non-SPF 7-week old outbred ISA Brown chickens. They were labelled with TCR2 and TCR3 and positively sorted, as described above, and were then stimulated (in triplicate) by incubation with immobilised anti-TCRα mAb AV33 (Parmentier et al., 1995) with or without anti-CD28 mAb AV7 (Young et al., 1994). Incubation was for 15 h at 41 °C in 5% CO₂, after which cells were harvested and RNA prepared using an RNeasy Mini kit (Qiagen) following the manufacturer's instructions.

2.3. Production of recombinant chIL-21 by COS-7 cells

COS-7 cells were routinely grown and passaged using standard conditions (Tregaskes and Young, 1997). Cells were transfected with either pCI-neo-chIL-21 or pCI-neo alone (for a negative control supernatant) using a DEAE-dextran-based method as previously described (Rothwell et al., 2001, 2004). Growth media were replaced with serum-free media 24 h post-transfection, and cells were then incubated for a further 72 h, prior to harvesting the supernatant containing the recombinant protein. Supernatants were stored at 4 °C prior to use.

2.4. IL-21 co-stimulation bioassay and flow cytometric analysis

Spleens and thymuses were removed from 8-week old SPF birds (outbred RIR or inbred line 7₂) *post mortem* and immediately placed in DMEM. Tissue was disrupted to release a single cell suspension, the lymphocytes were isolated over Histopaque 1077 (Sigma), washed twice and resuspended at 5 × 10⁶ cells/ml in DMEM containing 2 mg/ml BSA, 1% l-glutamine, 1 U/ml penicillin and 1 µg/ml streptomycin. 5 × 10⁵ cells were added to round-bottomed 96-well plates containing serial two-fold dilutions of recombinant (r) chIL-21 (ex-COS), in a final volume of 200 µl/well, in the presence or absence of 0.2 µg/ml ConA (Sigma), 4 µg/ml PHA (Sigma), 1 µg/ml lipopolysaccharide (LPS) (*E. coli* serotype 055:B5) (Sigma), or rchIL-2 (1/50 ex-COS supernatant; Rothwell et al., 2001). Samples were assayed in triplicate in five replicate assays in total. Cells were pulsed with ³H-thymidine (37 kBq/well, GE Healthcare, Little Chalfont, UK) for the final 6 h of a 48 h incubation at 41 °C, 5% CO₂. ³H-thymidine uptake was determined by liquid scintillation. Significance (taken as *P* < 0.05) of IL-21-treatment versus the relevant control was determined using Student's *t*-test.

2.5. Dendritic cell preparation and culture with IL-21

Chicken bone marrow-derived dendritic cells (chBM-DCs) were generated and expanded *in vitro* by culturing bone marrow precursors with GM-CSF and IL-4 as described (Wu et al., 2009b). On day 5 of culture, chBM-DCs were primed with different concentrations of rchIL-21 (ex-COS) (1:100, 1:250 or 1:500) for 18 h, prior to stimulation with LPS (100 or 500 ng/ml) for a further 8 h. RNA was isolated from the various DC cultures using an RNeasy Mini kit (Qiagen) following manufacturer's instructions; and expression of genes associated with activation and maturation of DCs analysed by real-time quantitative RT-PCR (qRT-PCR). Details of the qRT-PCR primers and probes are given in Table 1.

2.6. Real-time quantitative RT-PCR analysis

chIL-21 mRNA expression levels in different tissues and lymphoid cells were quantified by TaqMan qRT-PCR, using a

Table 1
Real-time quantitative RT-PCR probes and primers.

RNA target	Probe/primer sequence (5'–3')	Accession number
28S	Probe (FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA) F GGCGAAGCCAGAGGAAACT R GACGACCGATTGACGTC	X59733
IL-21	(FAM)-TGCTGCATACACCAGAAACCCTGGG-(TAMRA) AAAAGATGTGGTGAAAGATAAGGATGT GCTGTGAGCAGGCATCCA	AM773757
IFN- γ	Probe (FAM)-TGGCCAAGCTCCCGATGAACGA-(TAMRA) F GTGAAGAAGGTGAAAGATATCATGGA R GCITTCGCTGGATTCTCA	Y07922
IL-1 β	Probe (FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA) F GCTCTACATGCTGTGTGATGAG R TGTCGATGTCCCGCATGA	AJ245728
IL-6	Probe (FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA) F GCTCGCCGGCTTCGA R GGTAGGTCTGAAAGCGAACAG	AJ309540
IL-12 α	Probe (FAM)-CCAGCGTCTCTGCTTCTGCACCTT-(TAMRA) F TGGCCGCTGCAACG R ACCTCTCAAGGTGCACTCA	AY262751
CCR7	Probe (FAM)-TGAGGGTCAACATCGCTTTCAGCC-(TAMRA) F CATGGACGGCGGTAAACAG R TCATAGTCGCTGGTGACGTTGT	HQ269806
DC-LAMP	Probe (FAM)-TCTGCGCATTTCTTCCTGCTGTGC-(TAMRA) F CACACTTGCTCTGTGACCTTT R TGACACCCAGGGCCACTT	AM933592

F, forward primer; R, reverse primer.

well-described method (Avery et al., 2004; Eldaghayes et al., 2006; Rothwell et al., 2004). Sequences of primers and probes for chIL-21 and 28S RNA-specific amplification are given in Table 1. Probes were all labelled with the fluorescent reporter dye FAM at the 5' end and with the quencher TAMRA at the 3' end. The RT-PCR was performed using the TaqMan Fast Universal PCR master mix and one-step qRT-PCR master mix reagents (Applied Biosystems, Warrington, UK). Amplification and detection of specific products were performed using the 7500 Fast Real-Time PCR System (Applied Biosystems) with the following cycle profile: one cycle of 48 °C for 30 min and 95 °C for 20 s, and 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Results are expressed as 40-Ct, after normalising each sample using the Ct value for the 28S rRNA product for the same sample, as described previously (e.g. Avery et al., 2004; Eldaghayes et al., 2006; Rothwell et al., 2004).

3. Results and discussion

3.1. Characterisation of chicken IL-21 cDNA

The chIL-21 cDNA consists of 438 nucleotides, encoding an open reading frame of 145 amino acids (aa), with the predicted signal sequence (SignalP 3.0) comprising the first 19 aa. Comparison of the predicted aa sequence of chIL-21 with orthologous proteins from mammals, amphibia and fish (Fig. 1A) revealed 26–32% aa identity to mammalian IL-21, and 20% aa identity to *Xenopus* and fish IL-21. The four α -helical bundle structure of mammalian IL-21 is conserved in the chicken (Fig. 1) (Pspred; Jones, 1999), as are the four cysteine residues required for the formation of two disulphide bridges. A glutamine residue in aa position 129, which has been implicated in the binding to the IL-2 receptor γ -chain (Zurawski et al., 1993), is also conserved. There are two potential N-linked glycosylation sites (NXT at aa positions 83–85 and 134–136), as opposed to a single site in human and murine IL-21 (Parrish-Novak et al., 2000). The genomes of the zebra finch (Warren et al., 2010), turkey (Dalloul et al., 2010) and duck (<http://www.pre.ensembl.org>) have recently been assembled and a predicted IL-21 gene is present in each of these avian species. Comparison of the available avian IL-21 aa sequences shows much higher sequence identity amongst Aves (Fig. 1B) (53% aa identity to the zebra finch, 71% to the duck

and 78% to the turkey) than between the chicken and other classes of organisms. Alignment of the predicted avian IL-21 aa sequences with the chicken orthologue shows significant sequence differences at the 3' end of the gene, with both the zebra finch and turkey sequences 8 aa shorter than those of chicken and duck. For example, the current version of the zebra finch genome has two apparent isoforms of IL-21, one encoded by four exons and the other five. The first four exons of each splice variant are aa identical in sequence, yet only the first exon is in common between the two splice variants in the genome sequence.

There is conserved synteny of the IL-2 and IL-21 genes in mammals (human, mouse, cow, pig) (Parrish-Novak et al., 2000), birds (chicken, turkey and zebra finch) and *Fugu* (Bird et al., 2005), with these two genes lying close to each other on the same chromosome in each of these species.

3.2. Tissue distribution and cellular expression of chicken IL-21

Expression of chIL-21 mRNA in various tissues and cell subsets was analysed by qRT-PCR. As shown in Fig. 2A, chIL-21 is widely expressed, albeit at low levels, in most lymphoid tissues, including the gut; it is also detected in the lung, liver and skin. Highest expression was in Meckel's diverticulum, the lung, spleen and Harderian gland. The spleen, Meckel's diverticulum, the Harderian gland, and to a lesser extent the lung, are lymphoid tissues which are rich in plasma cells. In mammals, IL-21 plays a role in B cell differentiation into plasma cells (Ozaki et al., 2004; Ettinger et al., 2005) and one could postulate that the relatively high levels of IL-21 in these tissues could be driving the differentiation of B cells to plasma cells. Despite the fact that these tissues were isolated from specified-pathogen-free chickens, it is perfectly reasonable to suppose that these birds will be making antibodies to other pathogens or molecules. At the cellular level, IL-21 is expressed in CD4⁺ and TCR $\alpha\beta$ ⁺ (TCR2 and 3) cells, but not in CD8 α ⁺ or TCR $\gamma\delta$ ⁺ (TCR1) cells, and is up-regulated following mitogenic stimulation of CD4⁺ and TCR3⁺ cells (Fig. 2B). Specific stimulation of TCR $\alpha\beta$ ⁺ cells by TCR-cross-linking induced significantly greater levels of IL-21 expression ($P \leq 0.05$) (Fig. 2C) than non-specific mitogenic stimulation, but the addition of anti-CD28 did not enhance IL-21 expression. A very low level of IL-21 expression

A

Human	MRSSPGNMERIVICLMVIFLGTIVHKSSSQGD--RHMIRMRLD-IDQLKNYVNLVPEF	60
Cow	MR-WPGNMERIVICLMVIFSGTVAHKSSSQGD--RLFIIRLRQLD-IDQLKNYVNLDPPEF	59
Pig	MR-WPGNMEKIVICLMVIFSGTVAHKSSSQGD--RLIIRLRQLD-IDQLKNYVNLDPPEL	59
Mouse	-----MERIVCLVVIIFLGTVAHKSSSQGD--RLIIRLRHLID-IVEQLKIYENLDPEL	53
Xenopus	-----MANVILWCLLACSCCLIDAKLP-----STERCREIKKGIEEWIIKQANKTHPSF	50
Fugu	-----MKPLVLCFLFAVCCWCVADASADVSDKVKQORKTEEIR-ELGMVRRLOSKKML	55
Zebrafish	-----MKACVCFLLMCVLAQAEEESPK-----TLTISKVMN-ELKKIQOGLTNSNKTW	47
Chicken	-----MERMIIFCMLFFCFSMVLTAISP-----KAMKYKQLSK-TDHLKDVVKDKDEL	49
↑		
Human	LPAPED--DVETNCEWSAFSCF--OKAQLKSANT---GNNERTINVSIKKTKRRPPSTNAGRRO	116
Cow	LPAPED--DVKRHCERSAFSCF--OKVOLKSANN---GDNEKIINIITKQLKRRKLPATNTGRRO	115
Pig	LPAPED--DVQRHCEQSAFSCF--OKVELKSANT---GDNEKIINVIKQLKRRKLPPTNAGRRO	115
Mouse	LSAPQ--DVKGHCHEHAFAFCF--OKAKLPSNP---GNNKTFIIDIVAQLRRRLPARRGGKKQ	109
Xenopus	LHTPV--DVTABCEWSALNCFRYTSHLKEANG---QKEEDFKRNIRKVYLKRMHTPENKNG--	106
Fugu	STPSE--NIGDCCCLSAKCFRENFKETSLTD---LPQKKLYRSITNTITEKGLDFCDSKNS	113
Zebrafish	FNSPATNDLKDCCVASALECFRSKVLHLSVSDKELKKSQRSVHHEIRKSFIVDSVSCPTQETQ	110
Chicken	LHTPE--NPGDGCLLNAVTCF--QNGILRLQPK---NSQVNATFAKTVKLLRRPFLPVSEEH-	104
* * *		
Human	KHRLTC--PSCDSYEKKPPKEFLERFKSLQKMIHQHLS--SRTHGSEDS-	162
Cow	KHEVTC--PSCDSYEKKPPKEFLERLKSLLQKMIHQHLS-----	152
Pig	KHGLTC--PTCDSYEKKPIKEFLERLKSLLQKMIHQHLS-----	152
Mouse	KHIAC--PSCDSYEKKTPKEFLERLKWLLQKMIHQHLS-----	146
Xenopus	-----C-EICTKYPTQKEGDFLESMLLLQQTADYPTQTSG-----	142
Fugu	-----TC-QDCHSHPEEKAEEKFFDRNLNSLIQKVLKSSICHLMSLMF-	155
Zebrafish	KA--QC-KSCEAYTMVNSRTFVDNFKTLQKRSMPVKRRDLKSL----	151
Chicken	-----CESSCESYERKKPQEFNLNSFSKLMQKLFKNSTAERYGKSTI--	145
* * *		

B

Duck	MERMIIFCMLFFCFSMVLTAIPHAKMKYKQLKTKTIDQLKDVVKDKDAELLHTPENVEDC	60
Zebra finch	MKRMIFCMLFFCFSMVLTAISPRTLYKQIQKKIRDIESMVKDKDAELLHTPESLEEGC	60
Turkey	MERRIFCMLFFCFSMVLTAAPKAMKYKQLAKTIDQLKDVVKDKDVELLHTPENPEDGC	60
Chicken	MERMIIFCMLFFCFSMVLTAISPAMKYKQLSKTIDHLKDVVKDKDVELLHTPENEGDC	60
↑		
Duck	LETAVTCFQKGTILKLPKNSQVNSTEIKTVNLIKREILKDSGKQCESTCESYEKKNEKEEF	120
Zebra finch	LETAVTCFQKGTILKLPASSQENEAFAKAVRIVSKFTYKLPKEHCEFTCESYEKKNEKEEF	120
Turkey	LETAVTCFQKGTILKLPKNSQVNVITKTIVKILRRFIPDSGEHCESSCQSYERKKPQEEF	120
Chicken	LETAVTCFQKGTILKLPKNSQVNAFAKTVKILRRFIPVSEHCESSCQSYERKKPQEEF	120
* * *		
Duck	LQSFSLMLKKVITIRVDSFCLFFDLV	145
Zebra finch	LKGFENLMKMSRIHSS-----	137
Turkey	LNSFSKLMQKVIKIHQS-----	137
Chicken	LNSFSKLMQKLFKNSTAERYGKSTI	145
* * *		

Fig. 1. Alignment of the predicted chIL-21 amino acid sequence with (A) mammalian, amphibian and fish IL-21, and (B) other avian species, with reference to secondary structural features and functionally important residues. Shaded areas represent conservation of aa similarity – the darker the shading, the more conserved the residue across species. Dashes indicate gaps in the alignment. The predicted amino terminus of the mature chicken protein is indicated with an arrow. The four α -helices determined for hIL-21 are over-lined (A) and as predicted for chIL-21 (using Psipred; Jones, 1999) are underlined (B). Potential N-linked glycosylation sites are double-underlined. Four cysteines are conserved in all species and are indicated by asterisks, and the glutamine (Q) residue implicated in the interaction with the common γ chain receptor is also conserved (indicated by a solid triangle). The accession numbers of the sequences used are as follows: human, DQ645417; bovine, AB0703021; porcine, AB0703020; murine, DQ645418; *Xenopus*, EF533945; *Fugu*, AJ844554; zebrafish, EF143579; chicken, AM773757; zebra finch, XM_002188250; turkey, Pre-ensembl: ENSMGAT00000011849; duck, Pre-ensembl: ENSAPLT00000007179.

(40-Ct = 2.4) was detected in the ConA-stimulated CD8⁺ population, which could be attributed to contaminating CD4⁺ cells in the preparation, since the purity of cell sorting using the autoMACS Pro was approximately 90–95%. Similarly, a low level of IL-21 expression (40-Ct = 3.7) was also detected in the stimulated Bu-1⁺ cell subset, and this may also be due to contaminating CD4⁺ cells. However, although unlikely, in both cases it could represent induction of IL-21 expression in these two cell types. Overall, our data closely reflect what is seen in mammalian systems,

where IL-21 is expressed by CD4⁺ T and NKT cells (Parrish-Novak et al., 2000; Muneta et al., 2003; Coquet et al., 2007). It is not yet possible to determine whether IL-21 is expressed in chicken NKT cells, as these cells have yet to be described in the bird.

3.3. Biological activity of chicken IL-21

Recombinant chicken IL-21 was expressed in COS-7 cells and its effect on the proliferation of mitogen-stimulated splenocytes and

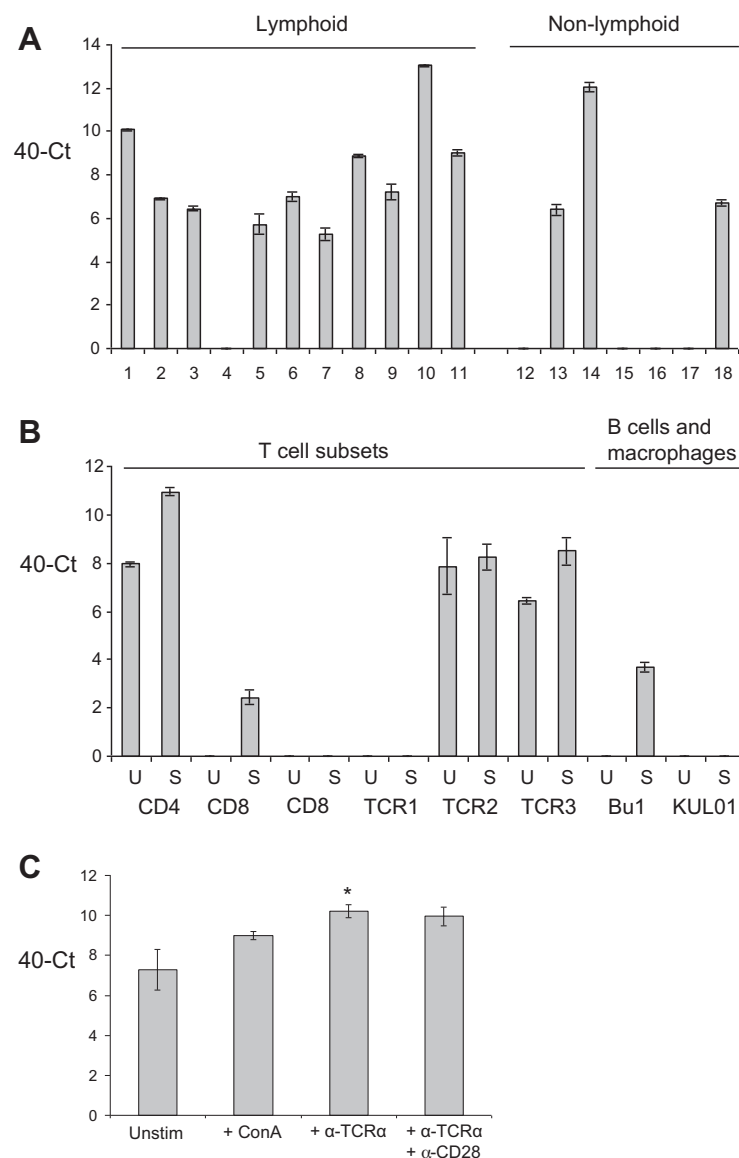


Fig. 2. Expression patterns of chIL-21 as measured by real-time qRT-PCR, with results expressed as 40-Ct values \pm SEM. (A) Chicken lymphoid and non-lymphoid tissues: 1, spleen; 2, thymus; 3, bursa of Fabricius; 4, bone marrow; 5, duodenum; 6, jejunum; 7, ileum; 8, caecum; 9, caecal tonsil; 10, Meckel's diverticulum; 11, Harderian gland; 12, kidney; 13, liver; 14, lung; 15, heart; 16, muscle; 17, brain; 18, skin. (B) Unstimulated (U) and stimulated (S) chicken splenocyte subsets. T cell subsets were stimulated with ConA, Bu1+ cells (B cells) were stimulated with PMA and KUL01+ cells (macrophages) were stimulated with LPS. (C) Splenic TCR $\alpha\beta$ + T cells were cultured in the presence or absence of ConA, anti-TCR α mAb, or anti-TCR α and anti-CD28 mAbs. Assays were carried out in triplicate, and representative data from one of four birds is shown. Student's *t*-test was used to determine significance ($P \leq 0.05$, indicated by an asterisk) of IL-21 expression, when compared to unstimulated and ConA-stimulated TCR $\alpha\beta$ + cells.

thymocytes was investigated. Fig. 3 shows that rchIL-21 significantly enhances the proliferation of splenocytes (Fig. 3A) and thymocytes (Fig. 3B) sub-optimally stimulated with mitogens (ConA or PHA) or LPS (splenocytes only) in a dose-dependent manner, at all dilutions shown, when compared to mitogen alone ($P < 0.05$). Recombinant chIL-21 alone also induced significant levels of proliferation in splenocytes ($P < 0.05$), but generally not in thymocytes. It also synergised with IL-2 to augment proliferation of splenocytes (Fig. 3C), but not thymocytes (data not shown). Similarly in mammals, IL-21 is a co-stimulatory factor for T cells.

In mammals, IL-21 has an inhibitory effect on DC activation and maturation (Brandt et al., 2003; Strengell et al., 2006). The effects of rchIL-21 on DC maturation were investigated by priming chBM-DCs (derived after culturing bone marrow cells for 5 days with GM-CSF and IL-4; Wu et al., 2009b) with rchIL-21, prior to activation with LPS. The mRNA expression levels of CCR7 (Wu et al., 2011) and DC-LAMP (Wu et al., 2010), typical markers of DC maturation, as well as the mRNA expression of the cytokines

IL-12 α , IFN- γ , IL-1 β and IL-6, were analysed by qRT-PCR (Fig. 4). All of these genes, with the exception of IL-12 α which was not expressed, were expressed at low levels in unstimulated chBM-DCs, and the addition of rchIL-21 to the culture media for the final 18 h made little difference to their expression levels (Fig. 4A). Upon LPS-stimulation of immature chBM-DCs, expression of the four cytokines was significantly up-regulated, as previously shown (Wu et al., 2009b, 2010), as was expression of DC-LAMP and CCR7 (Fig. 4B), indicating these are markers of DC maturation. In chBM-DC cultures that were primed with rchIL-21 and then stimulated with LPS, expression of all six genes was significantly down-regulated when compared to LPS-stimulated chBM-DCs in the absence of rchIL-21 (Fig. 4C). These data suggest that chIL-21 therefore has an immunosuppressive effect on the function of avian DCs, as it does in mammals.

Our data presented here on the cloning and initial characterisation of chIL-21 bioactivity indicate that, as with other chicken cytokines described to date, despite limited aa identity with their

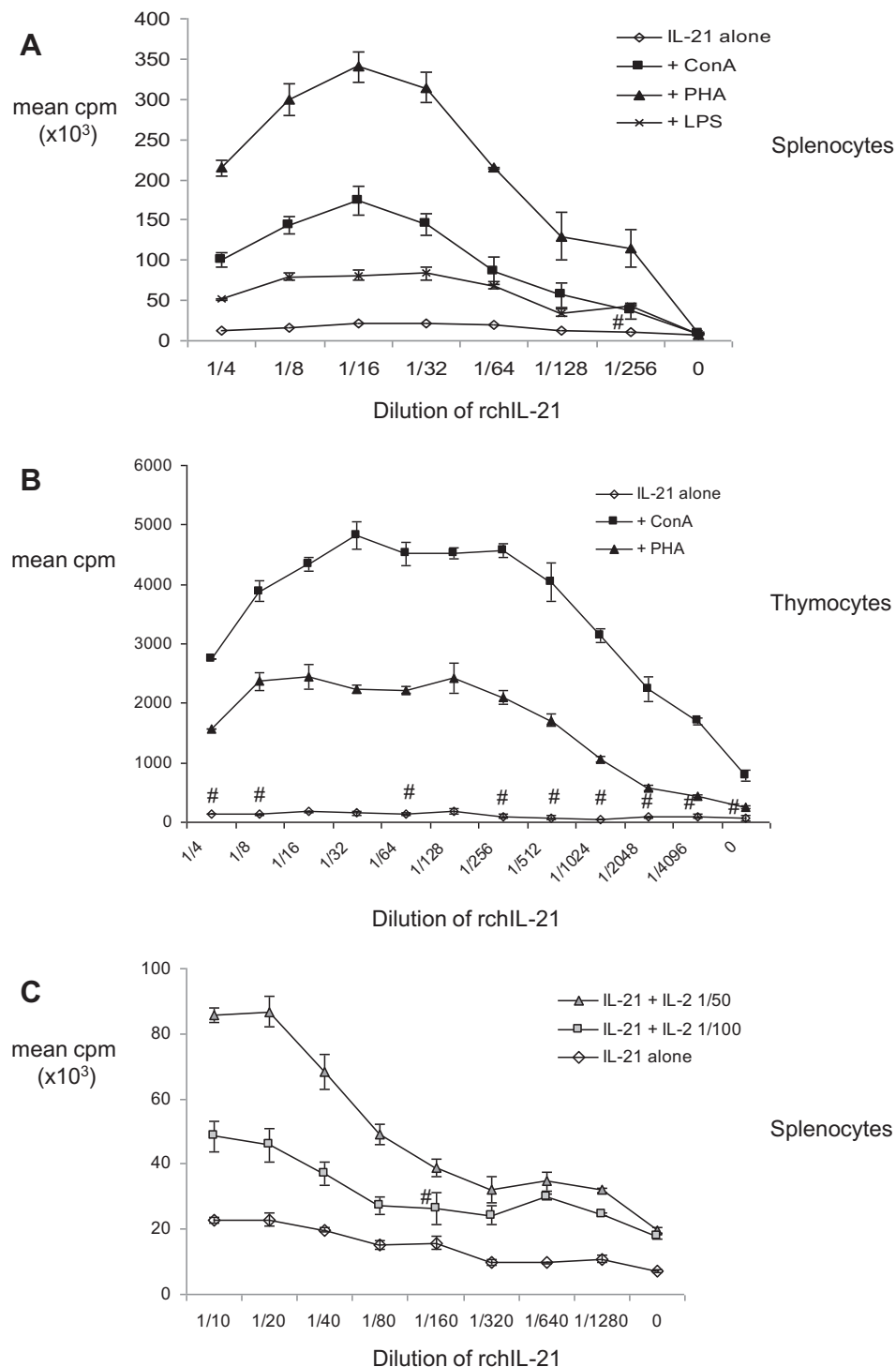


Fig. 3. Effect of IL-21 on the proliferation of (A) mitogen-stimulated splenocytes; (B) mitogen-stimulated thymocytes; (C) splenocytes co-stimulated with IL-2. Cells were unstimulated or stimulated with sub-optimal levels of mitogen (ConA or PHA) or LPS, or recombinant chIL-2 (ex-COS) at different dilutions, and co-cultured with two-fold dilutions of recombinant chIL-21 (ex-COS) for 48 h. Cell proliferation is expressed as counts per minute (cpm) by measuring incorporation of ^3H -thymidine. Error bars represent standard error of the mean (SEM) cpm of triplicate samples. Student's *t*-test was used to determine significance ($P < 0.05$) of IL-21 treatment, when compared to the relevant control. The # symbol indicates where IL-21 treatment did not significantly enhance proliferation. Data shown are representative of five separate experiments.

mammalian orthologues, there is conservation of bioactivity between the *Mammalia* and *Aves*. We have demonstrated that, as in mammals, chIL-21 is expressed by CD4 $^+$ TCR $\alpha\beta^+$ cells, acts as a co-stimulator of T cells, and has a negative regulatory effect on DCs. Further work is required to clarify if it plays a role in avian B cell differentiation. With the rapidly increasing number of characterised avian immune genes, including cytokines and cell surface

markers, we are now able to further dissect the avian immune response, and determine whether important new paradigms, to date only described in mammals, extend to the chicken. For example, identification of IL-21 and the preliminary bioactivity data presented herein provide additional and important new tools to begin to investigate whether the Th17 and Tfh cell lineages are present in the chicken.

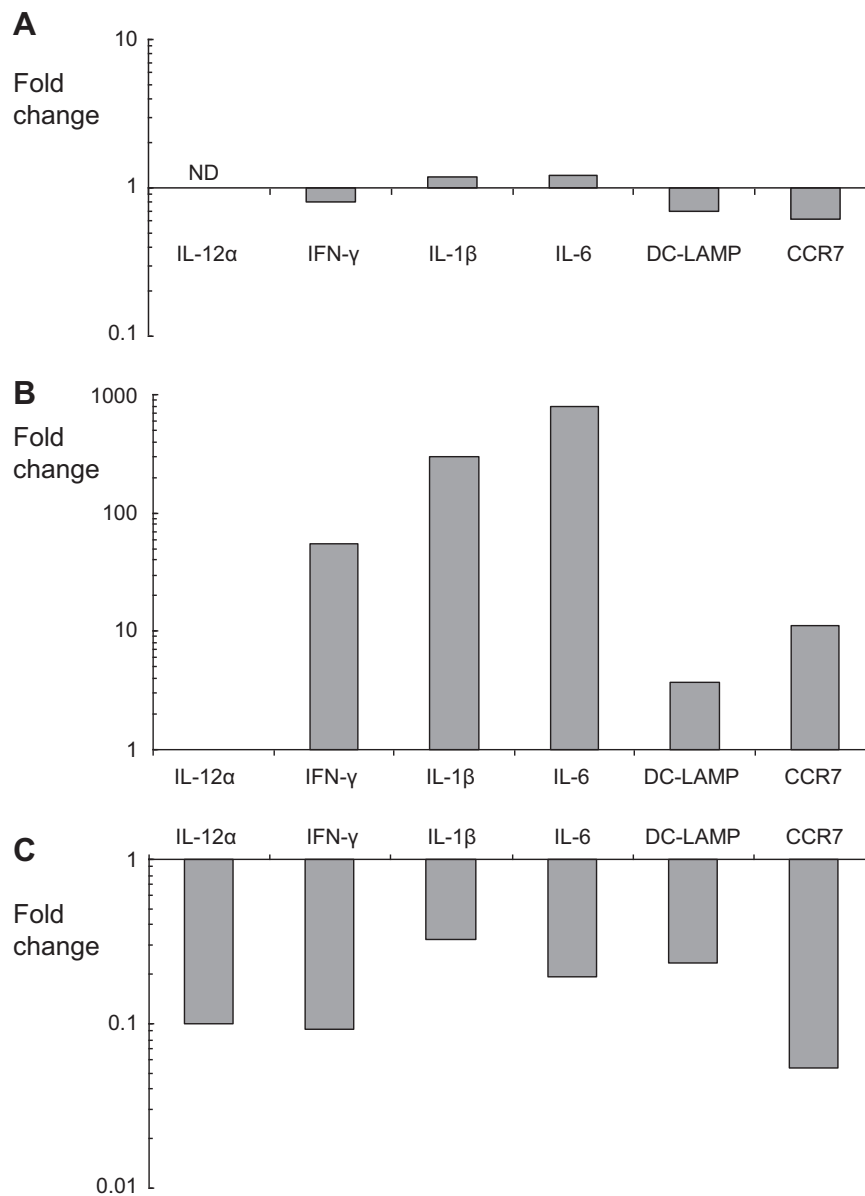


Fig. 4. Effect of IL-21 on dendritic cells (DCs), as determined by expression of genes associated with DC activation and maturation (including IL-12 α , IFN- γ , IL-1 β , IL-6, DC-LAMP and CCR7). mRNA levels were quantified by real-time qRT-PCR, and data are expressed as fold change as compared to the appropriate control. (A) Expression in immature DCs cultured with rchIL-21 (1:100 ex-COS) for 18 h as compared to immature DCs alone. (ND indicates IL-12 α was not detected in these samples). (B) Expression in DCs stimulated with 500 ng/ml LPS for 8 h as compared to unstimulated DCs. §, IL-12 α was not detected in unstimulated DCs (and therefore a fold-change value cannot be given), but was detected with a 40-Ct value of 8.1 when DCs were stimulated with LPS. (C) Expression in DCs primed with rchIL-21 (1:100 ex-COS) for 18 h followed by 8 h stimulation with LPS (500 ng/ml), as compared to DCs stimulated with LPS without IL-21 priming. Results are representative of three independent experiments.

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